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Review

Cell death induction by receptors of the TNF family: towards a molecular understanding

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1. Introduction

It has been known for a long time that programmed cell death (PCD) plays an essential role in the formation of a multicellular, differentiated animal [1]. A striking example is the development of the nematode *Caenorhabditis elegans*. The fully formed animal consists of 1089 cells, of which 131 are programmed to die. This system has provided a breakthrough in understanding of the molecular processes underlying PCD. Indeed, genetic studies first revealed the presence of genes needed for PCD, such as *ced-3* and *ced-4*, as well as other genes, such as *ced-9*, which counteract PCD [2]. In addition to its role in development, PCD occurs prominently in various immune processes, such as negative selection of T-cells in the thymus, elimination of overactivated lymphocytes in the periphery, etc. But PCD—or lack of it—has especially become a focus of considerable interest because of its relevance to many diseases [3]. For example, cancer cells have often become resistant to PCD-inducing stimuli, various lympho-proliferative and/or autoimmune diseases evade 'death' signals, etc. On the other hand, in some neuro-degenerative disorders such as Parkinson, Alzheimer or amyotrophic lateral sclerosis, one may wish to interfere with PCD.

Cells dying in the course of development are in fact fairly difficult to study, because the cell corpses are rapidly phagocytosed by neighbouring cells. Therefore, morphological and biochemical processes associated with PCD can best be studied in cell culture systems, and the typical events leading to cell death are usually referred to as 'apoptosis'. 'PCD' and 'apoptosis' are often used interchangeably, although the former has more a connotation of genetically predetermined, while the latter is more linked to morphological and biochemical changes [4]. The principal characteristics of apoptosis are blebbing of the plasma membrane, phosphatidylserine externalization, cytoskeletal disruption, accumulation and/or activation of transglutaminase, condensation of nuclear chromatin, fragmentation of nuclear DNA to approximately 50 kb segments, and subsequently to internucleosomal fragments; at later stages, cytoplasm and nucleus become compartmentalized and form membrane-bound apoptotic bodies, which are engulfed by neighbouring cells or infiltrated tissue macrophages [4]. Cell death by apoptosis is a very neat way to eliminate unwanted cells: no traces are left and the cell contents are never released or accessible to the immune system. Hence, there is no inflammation. This is in contrast to death by necrosis. Under these conditions, normally the cell swells and then, when membrane integrity comes under attack, the cell collapses like a balloon and the contents spill out into the extracellular milieu [5]. This may result in an inflammatory response.

Although PCD is sometimes referred to as 'cell suicide', in most systems studied the process of apoptosis is initiated by external inducers or treatments. Some examples are UV- or γ-irradiation, heat shock, oxidative stress, chemotherapeutic drugs (directed to topoisomerases like etoposide, to protein kinases like staurosporine, to DNA or its building blocks like nitrogen mustard and methotrexate, respectively, etc.), viral infection, loss of matrix attachment, glucocorticoids, growth factor withdrawal, soluble or membrane-bound cytokines like TNF or Fas/Apo-1 (CD95) ligand, and many others. In general, each cell contains precursors to apoptogenic mediators, as well as counteracting, protecting proteins. It is the balance between these two which determines the outcome after a given stimulus. It is remarkable how morphologically and biochemically similar apoptotically dying cells are, almost irrespective not only of the cell type, but also of the species. This suggests already that the molecular mechanisms of apoptosis are ubiquitous in nature and highly conserved in evolution. Indeed, various lines of evidence point to a universal, biochemical pathway. For example, the human anti-apoptotic protein Bcl-2 (to be discussed in a later section) can partially restore a defective *ced-9* function in *C. elegans* [6,7]. Equally important, in most systems of apoptosis experimentally studied, clear evidence for involvement of caspases was found. Caspases are a recently discovered, extended family of *C*ysteine-type proteases, which cleave after an *asp*artic acid residue; the *C. elegans* CED-3 protein and interleukin-1β-converting enzyme (ICE) are typical examples. Strong evidence for the involvement of caspases is the fact that usually the process of apoptosis can be interfered with by specific caspase inhibitors, such as zVAD-fmk or viral antagonistic pseudo-substrates (see below).

TNF and Fas/Apo-1 ligand play a prominent role in a variety of immunological, inflammatory and pathological conditions. Therefore, many studies on PCD have been done with TNF-R55 (CD120a) and Fas/Apo-1-induced death. Both receptors contain near their intracellular C-terminus a homologous region of about 90 amino acids, referred to as the 'death domain' (DD). Upon clustering, this DD is sufficient for sig-

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nalling cell death. TNF-R55-DD in addition leads to activation of the transcription factor NF-κB and to gene induction. Many of the induced genes are important for inflammation (IL-6, IL-8, ICAM-1, E-selectin), while activation of NF-κB is also involved in synthesis of resistance proteins, such as A20 [8]. Recently, other receptors of the same family have been cloned and shown to contain a DD-related sequence, such as DR3/WSL-1/TRAMP [9–11], chicken CAR-1 [12] and DR4 [13].

2. The TNF ligand and receptor families

The TNF receptor and ligand families are currently the object of intense research, covering a variety of different aspects of cellular signalling (reviewed in [14–20]). These ligand and receptor families, although large and continuing to increase in size, display rather homogeneous patterns of the structures involved in recognition. All members of the receptor family recognize their ligands through a shared sequence motif, a repetitive cysteine-rich structure found in different numbers in their extracellular domains. All but one of the ligands, viz. nerve growth factor (NGF), recognize their receptors through a shared structure composed of antiparallel β-strands, arranged in a 'β-jelly roll' topology. These similarities of structure are reflected in similar mechanisms of recognition and triggering. Apparently all members of the TNF ligand family occur and act in trimeric forms, allowing each ligand molecule to bind and cluster three receptors. Indeed, all TNF/NGF family members are activated as a consequence of juxtaposition of several receptor molecules and can thus be artificially triggered by their cross-linking with antibodies. Another common feature of the TNF ligand family is that they all act not only as soluble molecules, but also in association with the surface of their producing cells. Most of these ligands are produced as type-II transmembrane cell surface proteins from which the soluble forms are derived proteolytically. The only exception, lymphotoxin LT-α, although formed as a soluble secretable protein, can bind, as a subunit, to another member of the family, LT-β, and thus also acts as a cell-bound ligand.

In contrast to the rather homogeneous patterns of amino acid sequences and protein structures involved in ligand receptor recognition, the amino acid sequences of the intracellular domains of the TNF receptor family appear largely unrelated. This initially gave rise to the false impression that the receptors possess unrelated functions and mechanisms of action. With the increasing molecular understanding of the signalling by these receptors it was realized that this impression was wrong. It was realized, for example, that some previously undetected similarities of sequence exist between the intracellular domains. There appears to be even greater similarity of structure which is not reflected in sequence similarity, since a number of receptors with apparently dissimilar intracellular domain sequences have turned out to be capable of binding the same signalling molecules. In addition, there seems to be rather intense cross-talk between the different receptors through interaction of different signalling molecules that bind to them. These close interactions are reflected in a much greater similarity of function than was initially recognized.

This functional similarity also applies to the ability of the receptors of the family to trigger cytocidal effects. Detailed information about this activity is restricted mainly to the cytocidal function of TNF-R55, which signals to TNF cytotoxicity, and Fas/Apo-1, which signals to the cytocidal effect of Fas/Apo-1 ligand. Growing evidence indicates, however, that such effects can also be triggered by other receptors of the family. These include the two other TNF receptors known: TNF-R75 (CD120b), whose cytotoxic signalling [21,22] reportedly plays an important role in the self-destruction of activated CD8 lymphocytes (more so than that of TNF-R55) [23], and the LT-β receptor (which binds the complex formed between LT-α and LT-β) [24], and also DR3/WSL-1/TRAMP (a receptor closely related to TNF-R55) [9–11], the low-affinity NGF receptor [25,26], CD40 [27,28], and others. In this review we discuss the cytotoxicity induced by TNF-R55 and Fas/Apo-1, as these are the only receptors whose cytocidal function is at least partially understood at the molecular level.

3. Receptor-associated proteins involved in induction of cytotoxicity

In line with the multiplicity of functions mediated by TNF, there is growing evidence that the intracellular domain of TNF-R55, which signals for many of these activities, has the ability to bind and activate a variety of different signalling molecules. It is now known that the intracellular domain of Fas/Apo-1 also binds a number of different proteins. Most of the knowledge of these proteins and their function has been gained indirectly, for example by two-hybrid analysis of their interactive properties in transfected yeast, or by assessment of the interaction of the proteins when expressed in transfected cells at supra-physiological levels. The physiological relevance of the observations should therefore be carefully evaluated.

A major aid to assess the relevance of TNF-R55- and Fas/Apo-1-binding proteins to their cytocidal function was obtained when a distinct region involved in death induction was delineated within the receptor. As mentioned in Section 1, this homologous DD is about 90 amino acid residues long and was initially defined by exclusion. Whereas mutations within this region prevented death induction, mutations upstream or downstream of it had no such effect [29–31]. Positive evidence came from later experiments showing that cellular expression of transfected cDNA encoding this region alone is sufficient for death induction [32]. In the case of TNF-R55, clustering of the C-terminal domain, containing only 101 amino acids, was sufficient to elicit a full cytotoxic response and also NF-κB activation [33]. There are no sequence motifs in the DD that might allow it to exert direct cell-killing, enzymatic action. Moreover, several other proteins, some of them evidently unrelated to cell death induction, were also found to contain a similar domain [34]. The DD exerts its effects via interactive properties. As part of some proteins it was found to self-associate and also to be capable of binding to DDs in other proteins [35]. Preliminary data suggest that it can also bind proteins that do not contain a DD. The tendency of the DD in TNF-R55 and Fas/Apo-1 to self-associate apparently fortifies the interactions of receptor molecules imposed by ligand binding. Following their self-association, the DDs in the two receptors recruit and bind other DD-containing proteins, which serve as adapters in the signalling cascades. This binding is apparently made possible by exposure of the relevant regions in the receptors; these regions, according to mutational and NMR studies of

the DD in Fas/Apo-1, seem to be partly distinct from those involved in self-association of the receptors [36]. Fas/Apo-1 recruits a DD-containing adapter protein called MORT1/FADD [37,38], and TNF-R55 recruits a DD-containing adapter protein called TRADD [39]. Mutational studies indicate that the region upstream of the DD in MORT1/FADD is required for death induction by Fas/Apo-1 as well as by TNF-R55 [40,41]; moreover, since the DDs in MORT1/FADD and TRADD can bind to each other, it is assumed (so far with no direct evidence) that TNF-R55, when inducing death, recruits not only TRADD, but also its associated MORT1/FADD molecules. An additional DD-containing protein, called RIP [42], can also bind to TRADD as well as to MORT1/FADD [35]. It can also bind to a fourth DD-containing molecule called RAIDD [43] or CRADD [44], which in turn can interact with CASP-2 (CASP is a standard abbreviation for caspase). The observation that, upon overexpression, at least some of these proteins interact even in vivo, suggests that also they could be involved in a TNF-induced cascade of signalling to cell death.

Death induction is by no means the only function of the DDs in TNF-R55 and Fas/Apo-1. Detailed evidence implicates, for example, the DD of TNF-R55 in activation of NF-κB through its sequential interaction with TRADD and an adapter protein called TRAF2 (e.g., [41]). Conversely, despite the compelling evidence for a key role of the DD of the receptors in death induction, we cannot exclude the possibility that proteins associated with other regions in the intracellular domains contribute to the overall pattern of the death programme. There is already evidence for one such protein, viz. a protein tyrosine phosphatase, FAP-1, that binds to Fas/Apo-1 downstream of its DD and suppresses death induction by a mechanism still to be clarified [45].

Current notions of the function of signalling molecules activated by receptors of the TNF/NGF family are often based on tests that have certain shortcomings. The functional consequence of overexpression of a transfected protein may not be identical to that of the protein in a more physiological concentration. Overexpressed mutants may interfere with cellular functions by a mechanism that does not reflect a dominant negative effect, but rather events such as scavenging of other signalling proteins. It is therefore important to validate our concepts of the initial events in the signalling cascades by applying the acquired knowledge to analysis of what really occurs further downstream. In the case of cell death mechanisms, the immediate downstream event seems to be activation of specific caspases.

4. The caspases

As alluded to in Section 1, mutational studies of genes that control developmental cell death in nematodes [46] and the marked sequence homology observed between one of the genes identified in this way, *ced-3* [47], and a mammalian cysteine protease, ICE [48,49], recently opened the way to one of the most intensely studied aspects of current research on death mechanisms. Pursuant studies led to the identification of a whole family of proteases homologous to CED-3 and ICE which are widely expressed in mammalian cells. Although differing somewhat in exact substrate specificity, all of these proteases cleave proteins downstream of aspartate residues. They were accordingly dubbed 'caspases', for *c*ysteinyl *asp*artate-specific protein*ase* [50]. All caspases are synthesized as inactive precursors or zymogens. The precursor polypeptide consists of a prosequence (or a more complex prodomain in the case of CASP-8 and CASP-10), followed by a domain of approximately 20 kDa, often a small linker, and a domain of approximately 10 kDa. The prosequence is required for dimerization of the precursor [51]. Hetero- and/or auto-processing then leads to the formation of mature, tetrameric $(p20)_2(p10)_2$ [52]. Growing evidence points to a key role of caspases in a variety of different apoptotic processes (reviewed in [53–56]). There is even a tendency nowadays to consider that for a cell death process to occur in a programmed manner, caspase involvement is a prerequisite.

Several lines of evidence support the involvement of caspases in cell death induction by TNF and Fas/Apo-1:

(i) Receptor-induced cell death can be blocked by synthetic inhibitors of caspases. It can also be effectively blocked by two virus-produced proteins that act as caspase inhibitors, viz. the poxvirus serpine CrmA and the baculovirus p35 protein [57–60].

(ii) Caspases are produced as inactive precursors. As in other PCD processes, induction of cell death by TNF and Fas/Apo-1 has been found to result in rapid activation of certain caspases through their proteolytic processing [61–63].

(iii) Similarly, several proteins that serve as caspase substrates and have been shown to be cleaved in other apoptotic processes were also found to be cleaved at early stages of the death processes induced by TNF or Fas/Apo-1 [61,64–66].

As opposed to the rather solid evidence that caspases play an important role in processes of death induction, information about the nature of this role is still fragmentary. Also limited is information on the mechanisms of activation of caspases in the onset of the cell death process. Another major aspect of caspase function in which our knowledge is limited is the identity of the substrate proteins cleaved by caspases. Many of these substrates are presumably involved in the final events leading to apoptotic cell death (to be discussed in Section 6).

Another aspect of caspase action of which we have only partial understanding is the functional significance of their multiplicity in mammalian cells [55,56,67]. Although they share significant sequence homology, there are also clear differences of sequence between the caspases, enabling their classification into three distinct evolutionary groups. These differences are particularly pronounced in the regions upstream of the protease moieties (the 'prodomain' region). Growing evidence indicates that the prodomain regions regulate caspase activation and that their sequence heterogeneity allows activation of different caspases by different inducers. The most compelling evidence for this notion comes from studies of the way in which caspases are activated by TNF-R55 and Fas/Apo-1.

Attempts to uncover the mechanism by which TNF-R55 and Fas/Apo-1 trigger caspase action have led to the identification of three caspases whose prodomains can bind to adapter proteins interacting with these receptors. The prodomains of CASP-8 (MACH/FLICE/Mch5) [37,68,69] and CASP-10 (Mch4/FLICE2) [69,70] were both found to contain, in duplicates, a motif termed 'death effector domain' (DED) or the 'MORT' motif (also found upstream of the DD in MORT1/FADD), and to interact with MORT1/FADD through hetero-association of the DED motifs in the proteins. In fact, CASP-8 was cloned by screening for proteins that

interact with Fas/Apo-1 through MORT1/FADD. CASP-2 (ICH-1) was similarly found to interact through its prodomain with a homologous sequence in a protein called RAIDD [43] or CRADD [44]. The latter contains, downstream of its caspase-binding site, a DD that binds to the DD of RIP.

Recruitment of CASP-2, CASP-8 and CASP-10 to the receptors presumably results in their activation. Once activated, they are believed to be capable of processing and activating one or more of the other caspases found in the cell, thus triggering the death programme [71–73]. It should be stressed, however, that at present the evidence for this scenario is rather fragmentary. Overexpression of non-functional mutants of CASP-8 (in three cell lines) [37] or of CASP-10 (in one of the three) [70] was found to block both Fas/Apo-1- and TNF-induced death, suggesting that these two caspases play a crucial role in initiating the cytocidal effects of both receptors. CASP-8 was indeed shown to be recruited to Fas/Apo-1 upon triggering of this receptor [68,74]. So far, however, there is no evidence for its recruitment to TNF-R55, or for the recruitment of CASP-10 to either of the two receptors. Neither is there any evidence for involvement of CASP-2-RAIDD/CRADD interaction in TNF-R55 or Fas/Apo-1 action.

In cells that are resistant to Fas/Apo-1-induced cytotoxicity, these receptors are sometimes found to be incapable of recruiting MORT1/FADD and CASP-8 [74]. The molecular modifications that control the recruitment process are not known. A possible clue to their nature was provided by recent studies showing that CED-4, a death-regulatory protein in nematodes, can bind to the nematode caspase CED-3 and simultaneously to CED-9, a nematode homologue of Bcl-2, which can block cell death (cf. Section 5.3) [75,76]. Moreover, CED-4 was also found to be capable of binding to CASP-8, suggesting the possible existence of a mammalian CED-4 homologue that controls CASP-8 action by binding to it [75].

Many viruses have developed a strategy to avoid premature suicide of the infected cell. One example is expression of factors which interfere upstream in TNF-signalling and Fas/Apo-1-signalling interactions (dominant negative effectors) [77,78]. A second example are caspase pseudo-substrates which are powerful inhibitors of these enzymes, such as CrmA [79] and p35 [80]. A third class are factors which mimic the Bcl-2-type anti-apoptotic activity [81–83]. Similar factors of cellular origin may exist and modulate the apoptosis/anti-apoptosis balance; also molecules related to signal transduction factors, and formed for example by alternative splicing, may affect positively or negatively the final outcome [84,85].

5. Involvement of mitochondria and Bcl-2 family members in cell death

5.1. General

Early studies on apoptosis mainly focused on the nuclear events, which were indeed dramatic: DNA fragmentation and apoptotic body formation. But later on, the premise that the key steps in apoptosis occur in the nucleus was challenged. Removal of nuclei from cells still allowed induction of typical apoptotic events in the anucleate cytoplasts treated with anti-Fas/Apo-1 or staurosporine [86,87]. On the other hand, it has long been suspected that mitochondria play a key role in TNF-induced cell death. This hypothesis was tested by isolating cells devoid of mitochondrial DNA, and hence of a functional oxidative phosphorylation chain. In the case of TNF-induced necrosis, these cells were protected (see below), but they were still susceptible to (staurosporine-induced) cell death [87,88]. However, the latter experiments do not exclude a crucial role of mitochondria; indeed, mitochondrial proteins in mammalia are mainly nuclear-encoded, and cell lines lacking mitochondrial DNA and oxidative phosphorylation still contain mitochondria-like structures. These mitochondrial, DNA-deficient organelles maintain their membrane potential, and possibly could respond to triggers by the release of apoptogenic factors [89]. A second suggestion that mitochondria play a pivotal role in apoptosis follows from the localization of anti-apoptotic Bcl-2 family members. Bcl-2 is localized in the outer mitochondrial membrane, but also in the nuclear membrane and the endoplasmic reticulum. The main mechanism of action of Bcl-2 at the level of mitochondria will be discussed below (Section 5.3). The essential role of mitochondria was further revealed more directly by reconstructed, acellular systems of apoptotic processes. It is generally believed that the evident role of mitochondria is not a direct consequence of disruption of their energy-supplying function. In fact, RNA and protein synthesis usually continue unabated right until the onset of cell death.

5.2. Mitochondria-derived reactive oxygen intermediates (ROI): involvement in cytotoxicity

Matthews was the first to report that at least some types of cells treated with TNF showed gross abnormalities in the structure of their mitochondria; they looked swollen and had fewer cristae [90]. Furthermore, TNF-induced cell death was almost abrogated when the cells were kept in anaerobiosis, indicating a dependence on oxygen for cytotoxicity. In HEK293 or ME-180 cells, Wong et al. obtained protection by overexpression of mitochondrial manganous superoxide dismutase [91]. These results indicate that superoxide is formed as a result of TNF action and contributes directly to the cytotoxicity. MnSOD, which in many cells is induced by TNF, can be considered as a protective protein. However, these results cannot be generalized. In many other cell types, expression of MnSOD does not confer protection.

In early studies, including those of L. Old and colleagues [92], the tester cell line of choice was often the fibrosarcoma-derived L-cell line. Upon treatment with TNF, L929 cells die by necrosis [5] (it may be noted that there exist also L929 lines which die by apoptosis). In the case of necrotic death of L929 cells, the evidence that ROI are directly responsible for cytotoxicity is substantial. For example, the cells could be protected almost completely by preventing the electron flow from mitochondrial complex I and II to complex III, using the inhibitors amytal and thenoyltrifluoroacetone, respectively [93]. TNF treatment caused the formation of weird ultrastructural changes in the mitochondria of L929 cells. L929 sublines, selected on the basis of loss of mitochondrial DNA, became almost completely resistant to TNF-induced cytotoxicity. This is not in contradiction to the aforementioned statement that functional mitochondria-deficient cells were still susceptible to staurosporine-induced cell death, because in the latter system cell death proceeded by release of apoptogenic factors, as will be discussed below, while in L929 cells necrosis is due to ROI, which for their formation depend on electron flow to oxygen [94]. TNF leads to enhanced ROI production by the mitochondria, which can be quantitated on a per-cell basis [95]. Only some scavengers can efficiently

protect the cells from ROI. Presumably the reason is that they need to be present in the hydrophobic area where the ROI are formed; the most efficient is butylated hydroxyanisole (BHA). Addition of BHA to TNF-treated L929 cells almost immediately abrogates excess ROI formation and prevents further necrotic cell death [95]. The real target of the ROI effect which leads to the demise of the cell is not known; it may be some particular protein(s) or particular membrane peroxidation. Although L929 is an especially striking example of TNF-induced necrosis via ROI intermediates, it is certainly not an exception; there are many examples of tumour cell killing by ROI upon TNF treatment [96,97], and also some other agents can elicit ROI-dependent cell death [98,99].

5.3. *Mitochondrial permeability transition and modulation by Bcl-2 family members*

In many cells undergoing apoptosis, a fairly drastic drop in mitochondrial transmembrane potential ($\Delta\Psi_m$) is observed. A drop in $\Delta\Psi_m$ is due to permeability transition which allows small molecules (< 1500 Da) to leak out from the matrix [100]. This permeability transition is induced by nearly all stimuli which cause apoptosis, including TNF and anti-Fas/Apo-1. It is believed that the fall in $\Delta\Psi_m$ is a point of no return in the pathway to cell death [100]. What are the molecular events causing permeability transition? The precise molecular mechanism is still not clear, but a mass of evidence suggests that Bcl-2 and homologues are physiological regulators of permeability transition, and therefore an understanding of their mode of action is essential.

Bcl-2 was originally discovered by its overexpression in B-cell lymphoma/leukemia 2. In addition, Bcl-2 is frequently found to be (over)expressed in various carcinomas, lymphomas and leukemias, and is thought to play an important role in resistance to therapy [101–103]. Both Bcl-2 and its homologue Bcl-x_L are well expressed in embryonic tissues; in the adult, Bcl-2 is expressed in rapidly dividing cells, but not, for example, in the central nervous system, where only Bcl-x is expressed. Bax is another homologous protein which forms heterodimers with Bcl-2 and prevents the anti-apoptotic function of the latter. It seems quite likely that, at least in some tissues, the well-known tumour suppressor gene p53 exerts its function by induction of Bax [104]. It is of interest that overexpression of Bax can even cause death of yeast cells, but only under conditions where they require functional mitochondria [105]. In addition to CED-9 mentioned above, the Bcl-2/Bcl-x_L family further includes Bcl-W, A1 and Mcl-1 which, under appropriate conditions, can prevent apoptosis. On the other hand, the pro-apoptotic members include, in addition to Bax, Bik, Bak, Bad and Bcl-x_S (reviewed in [106]). Many viruses express anti-apoptotic functions, and some of these mimic Bcl-2. A striking example is the adenovirus E1B-19K protein, which only shows a minimal homology to Bcl-2, yet fulfils a similar anti-apoptotic function [81,102]. Bcl-2 and Bcl-x_L occur in the mitochondrial outer membrane, in the endoplasmic reticulum and in the nuclear membrane, but we have mentioned already that Bcl-2 can protect from PCD even in the absence of a nucleus [107]. Its occurrence on the mitochondrial membrane is rather patchy, suggesting interaction with special structures. Bcl-2 and homologues have a hydrophobic tail required for membrane association. Removal of this tail leads to cytosolic Bcl-2, which is far less effective in preventing apoptosis.

How does Bcl-2 promote cell survival? We have mentioned before that permeability transition and loss of $\Delta\Psi_m$ represent an irreversible step in the pathway to apoptosis. Hence, if Bcl-2 or Bcl-x_L protect cells, they must do so at a step before permeability transition, as has indeed been experimentally verified. In addition to form dimers with homologous family members, Bcl-2 also associates with the protein kinase Raf-1, and in this way drags Raf-1 to the mitochondrial membrane [108]. There, Bad becomes phosphorylated, resulting in its binding to the adapter protein 14-3-3, rather than to Bcl-x_L, thus blocking the pro-apoptotic function of Bad [109,110]. Also, Bcl-2 may itself become inactivated by phosphorylation [111]. However, the kinase responsible for phosphorylation of Bcl-2 and Bad is not Raf-1 itself. The modulating effects of protein phosphorylation on pro- and anti-apoptotic effects (Bcl-2 and Bax, respectively) have been recently reviewed [112].

More information on the presumed mechanism of action of Bcl-2 and homologues has come from recent structural studies. The 3D-structure of Bcl-x_L was shown to be similar to the structure of the pore-forming domains of bacterial toxins [113]. Indeed, Bcl-x_L can insert into lipid bi-layers and form cation-selective, ion-conducting channels [114]. Although this is the first demonstration of a physiological function of a Bcl-2 member, it is not yet proven that this property is responsible for the anti-apoptotic effect. Neither is there an attractive model which would explain how such a pore-forming molecule could prevent, for example, mitochondrial permeability transition.

What then is the trigger which causes the mitochondrial membrane transition pore formation, and which is counteracted by Bcl-2 and homologues? As we have mentioned before, a variety of apoptogenic stimuli converge to effect the collapse of the mitochondrial membrane potential. The signalling pathway emanating from TNF or anti-Fas/Apo-1 triggering can be inhibited by CrmA and zVAD-fmk, specific inhibitors of caspases. It is conceivable that the activated CASP-8 released from the receptor complex or downstream-activated caspases could directly act on the mitochondria. In fact, important new findings support such a hypothetical scenario. And this evidence comes again from the elegant *C. elegans* system. In this organism, it is well known that in addition to CED-3 there is also a requirement for the protein of the *ced-4* gene in order to execute cell death. The mammalian homologue of CED-4 has so far not been found. But recently it was discovered that the CED-9 protein, presumably localized in the mitochondria, physically interacts with CED-4, which itself interacts with CED-3. Mutational analysis indicates that this binding correlates with functionality [76,115]. Moreover, CED-4 can also physically interact with mammalian CASP-1 and CASP-8. In this way, a possible link between the mitochondria and the receptor-signalling system of Fas/Apo-1 and TNF-R55 has been established [38]. It is also of interest to note that, although several pathways leading to apoptosis converge at the events occurring at the mitochondrial membrane, which often can be inhibited by Bcl-2, the upstream requirement for caspases is not a universal phenomenon. For example, signals to mitochondria that are coming from the TNF receptor or from Fas/Apo-1 are blocked, as mentioned above, by caspase inhibitors, while the pathways stimulated by irradiation, by chemotherapeutic drugs or by peroxide are not affected [116].

5.4. Mitochondria-driven steps in signalling

After Fas/Apo-1 cross-linking, activation of CASP-1-like enzymes precedes CASP-3-like activation and nuclear apoptosis. Inhibition of this CASP-3 activity prevents the downstream terminal phase, during which various substrates are cleaved. CASP-3 and homologues exist in a precursor form in the cytosol, and require proteolytic activation for functioning. The role of the mitochondria in this activation is not yet known in detail, but some interesting observations have been made using cell-free systems. Lazebnik et al. [117] were the first to show that it was possible to make an extract from cells in the condemned phase of apoptosis, which could induce in isolated nuclei morphological and biochemical features that were typical of a nucleus in an apoptotically dying cell. Parallel studies with *Xenopus* oocyte extracts showed that an essential component responsible for caspase activation and apoptotic effects in cell-free extracts was in fact the mitochondria [118]. Fractionation of the apoptogenic extract revealed that there are at least two factors required for CASP-3 activation, one of which, surprisingly, was cytochrome *c* [119]. By itself, cytochrome *c* cannot cleave or activate recombinant CASP-3 in vitro. It is likely that it interacts with (a) pre-existing cytoplasmic factor(s) which subsequently mediate(s) the cleavage of zymogens. Cells undergoing apoptosis were found to have elevation of cytochrome *c* in the cytosol and a corresponding decrease in the mitochondria. Overexpression of Bcl-2 or even exogenous addition of recombinant Bcl-2 to isolated mitochondria prevented the efflux of cytochrome *c* [120]. Remarkably, cytochrome *c* release was unaccompanied by changes in $\Delta\Psi_m$ [116]. It is interesting to note that in *C. elegans* the messenger coding for CED-9 is in fact polycistronic and also leads to synthesis of a cytochrome-*b*560-related protein [7]. Another factor, also released from mitochondria, gives a better view of how it could initiate CASP-3-like activation. It has been reported that mitochondrial permeability transition also results in a Bcl-2-inhibitable release of a ~50 kDa intermembrane protein that was sufficient to cause nuclear apoptosis [121]. The activity of this protein, referred to as AIF or 'apoptosis-inducing factor', was inhibited by zVAD-fmk, but not by any of the other caspase inhibitors. Moreover, AIF causes CASP-3 activation in vitro, connecting the mitochondrial phase of apoptosis with a downstream caspase activation pathway. Whether cytochrome *c* is a co-factor in this process or whether it is involved in a parallel pathway, is at present unknown.

6. Caspase substrates and their involvement in the final events of the apoptotic process

6.1. General

The end of the apoptotic process is characterized by a common degradation phase during which cells acquire the biochemical and morphological features of end-stage apoptosis. While the morphological changes are quite well characterized, the biochemical basis for these dramatic events is still unclear, although caspases seem to play an important role also at this stage.

Four kinds of caspase substrates, each mediating a distinct set of functions, can generally be distinguished: (i) the first caspase substrate to be identified was the IL-1β precursor, cleaved by ICE (CASP-1); processing of this precursor (and other substrates of the same category [122–124]) is not involved in apoptosis, but rather contributes to the induction of inflammation; (ii) there is considerable evidence that autoprocessing plays an important role in activation and maturation of caspases [52]; furthermore, at least some of the caspases are involved in a cascade of sequential activation [71,72]; (iii) structural proteins, such as lamin or actin, the cleavage of which is likely to contribute to the disassembly of cellular structures during the death process; (iv) other proteins with a variety of activities. Some of the proteins of the last group have enzymatic activities that may help to repair damage in the dying cell. It has been suggested that their cleavage, and subsequent inactivation, results in enhancement of the death process [125]. Since none of the proteins found to be cleaved by caspases has been shown to serve a vital function, it is difficult to understand how the activation of these enzymes results in cell death. Our current knowledge of the identity of the caspase substrates is based solely on chance identification. A systematic analysis of the pattern of caspase substrates may be required in order to determine how the activities of these proteases contribute to the induction of death and the extent to which their contribution is assisted by other activities, induced independently by the TNF receptors and Fas/Apo-1.

6.2. Nuclear changes

As mentioned in Section 1, the nuclear events of apoptosis include chromatin condensation, DNA fragmentation to domain-sized fragments (30–50 and 200–300 kb) and then to oligonucleosomes (180–200 bp), cleavage of specific target proteins, and ultimately budding of nuclei into membrane-enclosed apoptotic bodies. Several endonucleases have been proposed to be involved in the degradation of DNA during apoptosis (for a review on nucleases in apoptosis, see [126]). One of the nuclear proteins which is cleaved during apoptosis is the DNA repair-associated enzyme, poly(ADP-ribose) polymerase (PARP) [127]. Enzymes which can cleave PARP include CASP-3, CASP-7, CASP-8 and CASP-9 [55]. The DNA-dependent protein kinase is another enzyme which is involved in DNA repair and which is also cleaved by CASP-3-like proteases [128]. CASP-6 cleaves the nuclear lamins [129], intermediate filament proteins that form a meshwork beneath the inner nuclear membrane and provide a framework for the attachment of chromatin to the nuclear envelope. Because this meshwork is important for the stability of the nuclear envelope, it makes sense that lamin cleavage is required for disassembly of the nucleus into individual apoptotic bodies. Another substrate that is cleaved after Fas/Apo-1 and TNF treatment, is the 70-kDa component of the U1 small ribonucleoprotein [65]. Cleavage of this protein may play a role in regulating RNA splicing and in loss of chromatin organization during apoptosis [130].

PITSLRE kinases are a superfamily of protein kinases related to the master mitotic protein kinase, cdc2. Induction of apoptosis via the Fas/Apo-1 receptor in human T-cells has been shown to be correlated with proteolysis and increased activity of PITSLRE kinases [131]. Furthermore, cleavage of at least some isoforms of PITSLRE kinases during TNF-induced apoptosis has recently been shown to be mediated by CASP-3 or a close homologue [66]. Ectopic expression of the smallest member of this superfamily has previously been shown to induce apoptosis [132]. In addition, deletion of the PITSLRE gene complex and complete loss of expression of

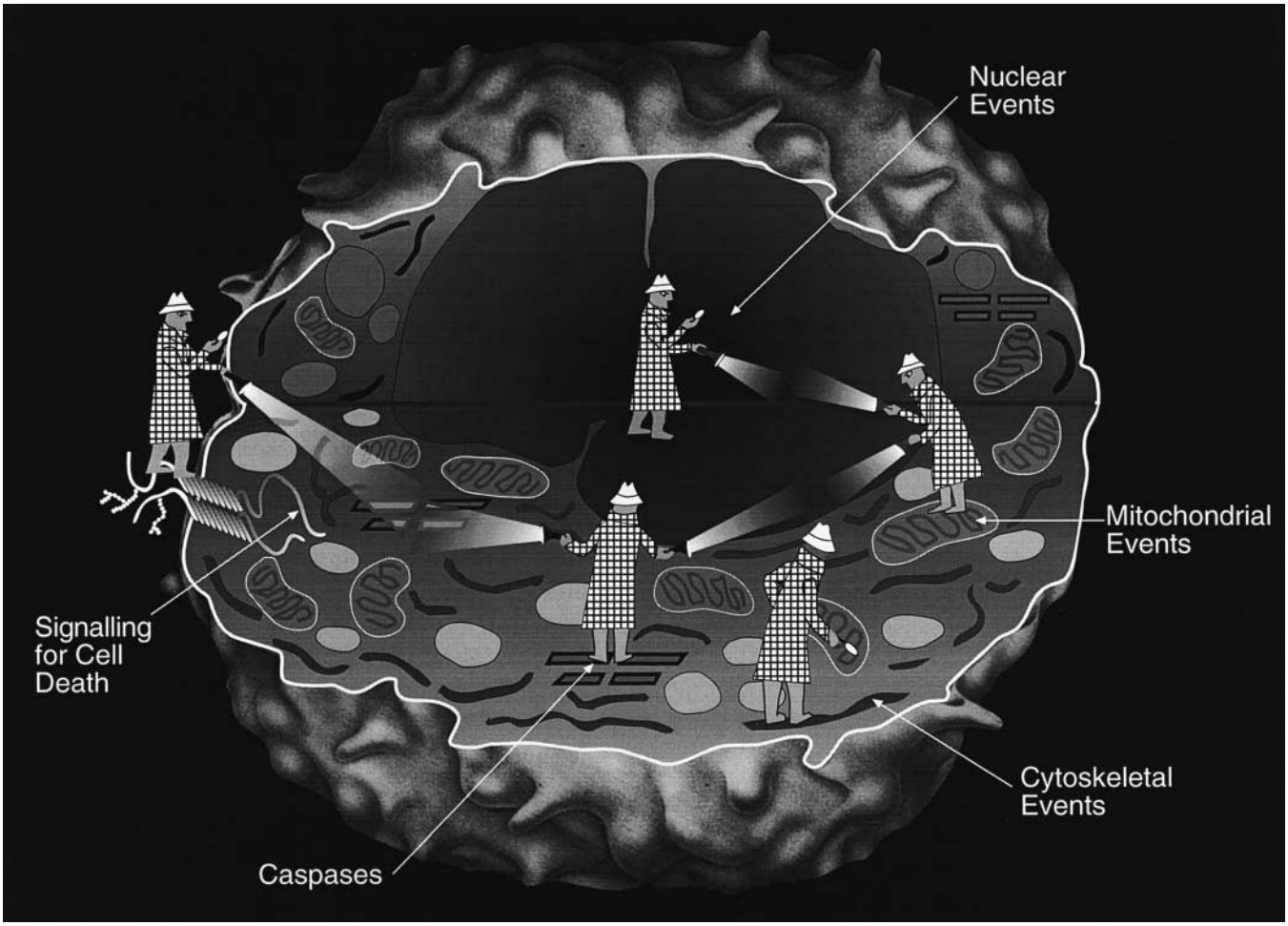

Fig. 1. A cartoonist's view of the cell death field.

specific isoforms occur in many neuroblastoma cell lines and are frequently observed in human cancers [133]. Therefore, PITSLRE kinases may have a role in the apoptotic effector phase, rather than being involved in the final degradation phase. Another kinase which has recently been implicated in nuclear phenotypic changes associated with apoptosis induced by anti-Fas/Apo-1 and TNF is protein kinase C δ. Cleavage of the latter by CASP-3 is associated with activation of the kinase function [134].

6.3. Cytoplasmic changes

One of the most noticeable morphological features of apoptosis is fragmentation of the cell into apoptotic bodies. A rearrangement of the microfilament network of the cell must occur during this process and may be responsible for some of the observed changes. Cleavage of α-fodrin and actin during apoptosis induced by Fas/Apo-1 and several other stimuli has been shown to be mediated by caspases [64,135]. Cleavage of these structural proteins may be related to the process of blebbing observed during the final stages of the apoptotic process.

At the onset of apoptosis there is a large increase in transglutaminase activity which is responsible for cross-linking of proteins. This will stabilize the cytoplasm of dying cells, preventing the leakage of harmful and pro-inflammatory or immunogenic, intracellular elements into the external milieu.

6.4. Cell membrane alterations in apoptosis

During apoptosis, there are extensive cell membrane alterations; the cell detaches from neighbouring cells and from the extracellular matrix. In addition, apoptotic cells display cell-surface markers (e.g., thrombospondin, CD36) that ensure their recognition and removal by phagocytic cells. Phagocytic receptors also recognize changes in membrane sugars and lipid composition (exposure of negatively charged phosphatidyl serine on the outer leaflet) [136]. Activation of sphingomyelinase and the sustained elevation of ceramide levels observed after several hours of TNF treatment can be prevented by the caspase inhibitor, CrmA [137], suggesting that ceramide may function as a downstream effector of apoptosis, rather than as an upstream primary signalling switch.

7. Concluding remarks

One can afford to propose models when knowledge is comprehensive, or when almost nothing is known. In the field of cell death research we are now at an intermediate stage, where too little is known for detailed models and too much for indulgence in pure hypotheses. Allegorically, applying the metaphor of the 'black box' often used to represent large gaps in knowledge, our current molecular understanding of cell death could be portrayed as the emergence of several point-light sources that illuminate scattered patches in the

black box, but do not yet merge to give a clear view of the box as a whole (see Fig. 1).

Our state of the understanding of death induction by receptors of the TNF family corresponds to our general state of knowledge of PCD. The information on some of the molecules that apparently participate in these common mechanisms is very limited. There is a reason to believe, for example, that cellular inhibitor-of-apoptosis proteins (c-IAPs) play a role in the control of cell killing by the TNF receptor family. Two c-IAPs (c-IAP1 and c-IAP2) were found to bind to the TNF-R75-associated protein, TRAF2 [138,139], and to be recruited with it to TNF-R55 upon receptor activation [140]. Little is known, however, about how c-IAPs act and how they actually affect TNF cytotoxicity. Another example concerns the involvement of specific oncogenes in TNF cytotoxicity. There is conclusive evidence that the level of c-Myc expression affects cell vulnerability to TNF cytotoxicity, at least in some cell systems [141–143]; however, in the absence of information on the mechanisms by which c-Myc affects cell viability, this information provides no more than a starting point for future exploration.

The focus of this review was on four sources of information that did yield significant knowledge about the mechanisms of cell death induction. One of them is unique to the study of death induced by the TNF receptor family, namely the sequential analysis of signalling cascades activated by the receptors. The other three are common to the cell death field: studies of the caspases, of the Bcl-2 family, and—presumably associated with it—of the changes that occur in the mitochondria during cell death. As described above, each of these directions of research has provided a view of a whole series of molecular changes that participate in death induced by Fas/Apo-1 or TNF-R55. The large remaining gaps still leave room for doubts as to the validity of our present notions. Gaps are particularly noticeable in the zones of overlap between the mechanistic domains illuminated by each of the four directions of research. How, for example, does the signalling cascade initiate the changes that occur in the mitochondria? What is the exact contribution of the mitochondrial changes and of the Bcl-2 family members to caspase activation? The present-day quest for the Holy Grail therefore is directed to the identification of the mammalian homologue of the *C. elegans ced-4* gene product, which may shed more light on some of these questions.

To advance further, it is important to define clearly those points requiring elucidation. The extensive phenomenological knowledge gained over the 8 years of study of the death processes activated by Fas/Apo-1 [144,145] and the almost 30 years of study of death induced by TNF/LT [92,146,147] form a solid basis for delineation of the gaps in our understanding of the mechanisms involved. Here are a few of the questions that may arise when attempting to reconcile these old phenomenological data with our current molecular understanding:

(i) *The kinetics of death.* It was surprising to find that CASP-8 and CASP-10 associate directly with the Fas/Apo-1 and TNF-R55 signalling complexes, and thereby are apparently activated immediately upon triggering of the receptor. This finding seems inconsistent with the long-known fact that there is a time lag, of at least several hours, between the initial triggering of TNF-R55 or Fas/Apo-1 and the death process (e.g., [148]). The molecular basis for this gap is unknown.

(ii) *Regulation of sensitivity to the cytocidal activity of the receptors.* Although the major constituents of the death-signalling complex have been identified, we still lack understanding of the quantitative determinants of the process. Cells that express the same amounts of TNF-R55 or Fas/Apo-1, their adapter proteins and the various caspases can differ substantially in their vulnerability to the cytocidal effect of the receptors. Several lines of evidence indicate that this variation involves the activity of 'protective proteins' that render cells resistant to the cytocidal effect. Moreover, there are indications that at least some of these protective proteins are induced by TNF itself through activation of NF-κB, thus exerting a negative feedback control of TNF function (reviewed in [149]). Although several TNF-induced proteins capable of providing partial resistance in some cells against TNF cytotoxicity have been identified [91,150,151], it seems that the identity of the major determinants of vulnerability to death induction by the receptors still remains to be defined. Furthermore, many malignant cell lines become much more sensitive to TNF after co-treatment with IFN-γ, and the molecular reason for this synergy also escapes us.

(iii) *Relationship between the cell-killing activities of Fas/Apo-1 and TNF-R55.* Several comparative studies of cytotoxicity induction by Fas/Apo-1 and TNF-R55 indicate that, despite their close structural similarity, their mechanisms of death induction are somewhat different. This is manifested in different morphological features and different ways of modulation of the death processes by various pharmacological agents and cytokines [152–155]. Such differences were observed, not only when comparing the way death occurs upon triggering of the two receptors in different cells, but even when death was induced by the two receptors in the same cell, suggesting that they reflect differences in the mechanisms of action of the two receptors. Our present information on the mechanisms of signalling to death provides few clues to the nature of this difference. The components of the signalling complexes so far found by mutational analysis to be specifically involved in the induction of death, namely MORT1/FADD, CASP-8 and CASP-10, seem to be equally involved in death induction by each of the receptors [37,38,41,70]. As mentioned above, clustering of TNF-R55 leads via TRADD→Traf2 to rapid NF-κB activation and gene induction; but differences between TNF-R55 and Fas/Apo-1 in the phenomena of death induction persist even in the presence of protein synthesis inhibitors.

Perhaps the most important recent lesson of the death-inducing activity of the TNF ligand family is that these mediators of immune killer cell function employ the same molecules and mechanisms as those involved in all other PCD processes: caspases, mitochondrial functions, etc. Thus when the above and related questions are investigated, the knowledge gained in connection with the cell-killing activity of TNF-R55 and Fas/Apo-1 need not necessarily be restricted to this particular field, but may promote our understanding of cell death in general.

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